# REVERSIBLE DINITROPHENYLATION OF GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE. IDENTIFICATION OF THE LABELED SITE

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## 1. Introduction

The thiolytic removal of dinitrophenyl (DNP*) groups from sulfhydryls, imidazoles and phenolic hydroxyls [1] has converted dinitrophenylation into a method for the reversible labeling of these functional groups in proteins. The major advantage of this technique lies in the fact that it combines two useful features: a) the label can be easily removed under conditions which do not cause any irreversible damage to the protein, b) its binding is stable enough to withstand the manipulations required for fragmentation and sequence studies, so that the exact location of the labeled amino acid can be identified.

We have recently demonstrated the mildness of the procedure by applying it to GAPD (EC 1.2.1.12) [2]. Stoichiometric dinitrophenylation of a cysteine residue in apo-GAPD inactivated the enzyme and subsequent thiolysis of the label resulted in full restoration of the catalytic activity. We wish to report here the isolation and sequence determination of a pentapeptide fragment from GAPD containing the labeled cysteine.

## 2. Materials and methods

GAPD (from rabbit muscle) was purchased from Sigma and assayed as described by Velick [3]. Freshly dissolved enzyme crystals had a specific activity $k_2/E = (1.8 \pm 0.2) \times 10^6$ ($k_2$ is the second-order rate constant expressed in l.mole$^{-1}$ min$^{-1}$ and E is the enzyme concentration in mg/ml). Apo-GAPD (the $NAD^+$-free enzyme) was prepared by the method of Havsteen [4]. The preparation used had $A_{280nm}$: $A_{260nm}$ ratios between 1.85 and 1.95. Protein concentrations were determined spectrophotometrically at 280 nm, using absorbance coefficients of 1.00 cm$^2$ mg$^{-1}$ for GAPD and 0.829 cm$^2$ mg$^{-1}$ for apo-GAPD [5]. The molecular weight of the enzyme was taken as 140,000 (4 subunits) [6, 7].

Uniformly labeled $^{14}$C-FDNB was purchased from the Radiochemical Centre, England. The specific radioactivity of FDNB solutions was determined as described previously [2]. Radioactivity was measured in a Packard Model 3003 Tri-Carb liquid scintillation spectrometer.

Protein hydrolysis was carried out with 5.7 N HCl (22 hr, 110°) in evacuated sealed tubes. Chromatography and high-voltage electrophoresis [2] were used for the identification of S-DNP-cysteine. Amino acid compositions were determined by the method of Spackman, Moore and Stein [8], *N*-terminals were determined by the Dansyl method [9] and sequence analysis was performed by the use of carboxypeptidase A [10].

## 3. Results

Rabbit muscle apo-GAPD (420 mg, 3 μmoles) was reacted with $^{14}$C-FDNB (16.5 μmoles; specific radioactivity 2.75 × 10$^5$ cpm/μmole) in a buffer composed

* *Abbreviations:*
DNP : 2,4-dinitrophenyl
FDNB: 1-fluoro 2,4-dinitrobenzene
GAPD: D-glyceraldehyde 3-phosphate dehydrogenase.

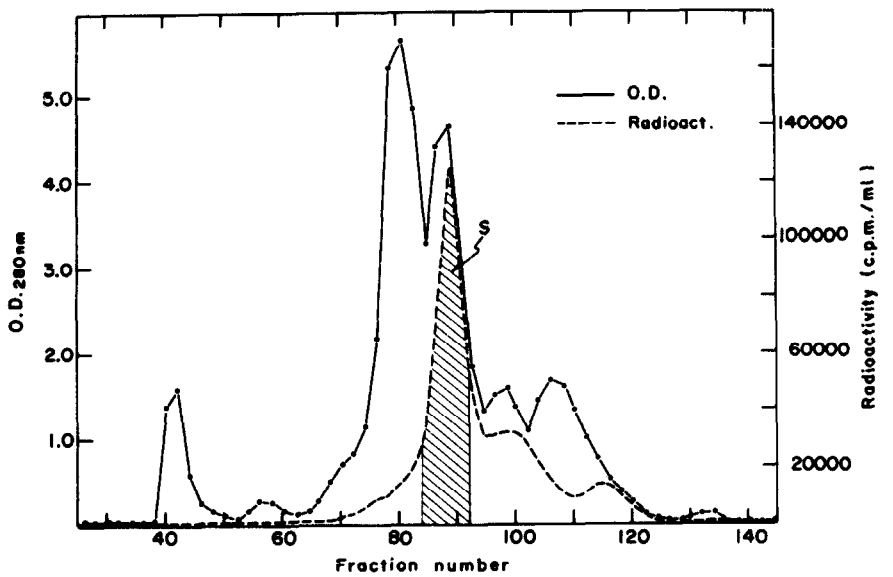


Fig. 1. Elution pattern of the peptic digest of S-DNP-apo-GAPD. The column (Sephadex G-25 fine, 180 X 1.5 cm) was equilibrated at 22° with 2% acetic acid. Fractions of 3.2 ml were collected and their absorbancy (——) as well as their radioactivity (- - -) were monitored.

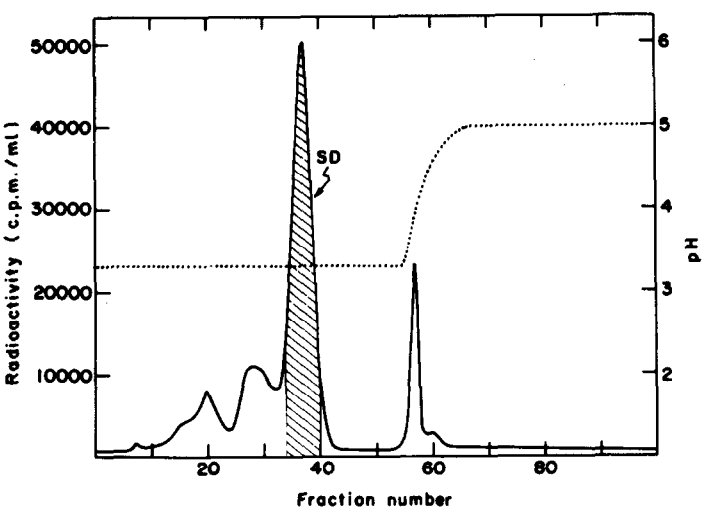


Fig. 2. Fractionation of the major radioactive peak from the Sephadex column (S) on Dowex 50-X8. The column (0.9 X 35 cm) was water jacketed and eluted at 50°, first with a 0.2 M pyridine acetate buffer, pH 3.3 and then with 2 M pyridine acetate, pH 5.0. The flow rate was 50 ml/hr. Fractions of 2.5 ml were collected and their radioactivity (——) as well as their pH (.....) were measured.

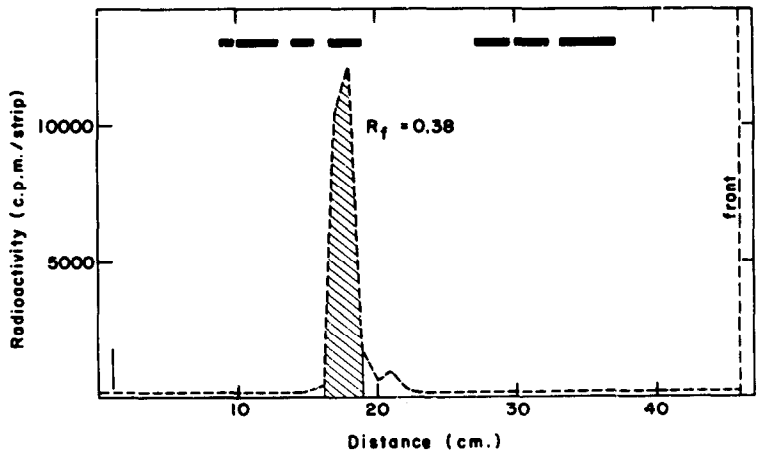


Fig. 3. Purification of the major radioactive peak from the Dowex column (SD) by preparative paper chromatography. The pool of the SD fractions was dried in vacuo, dissolved in 1.5 ml of 2% acetic acid and applied on Whatman paper No. 3 in a strip, 15 cm long. The chromatography was run for 16 hr in butanol-acetic acid-water (4:1:4, upper layer). The radioactive peptide was located by cutting a strip of paper (0.5 cm wide) along the chromatogram, dividing it into pieces (1 cm long) and measuring their radioactivity. Horizontal bars indicate ninhydrin-positive materials.

of sodium pyrophosphate ($3 \times 10^{-2}$ M) and EDTA ($10^{-3}$ M), pH 8.1. The reaction was conducted at a protein concentration of 40 mg/ml and allowed to proceed at 22° for 30 min, then dialyzed twice against 3 l of 0.02 N HCl. A transparent yellow gel was formed which was dissolved by addition of 20 ml of 0.02 N HCl to the dialyzed sample. Under these conditions the extent of incorporation of $^{14}$C-DNP groups was between 2.8 and 3.4 moles of $^{14}$C-DNP groups per mole (140,000 g) of enzyme.

Table 1
Sequence of peptide SD after purification by paper chromatography.

| Suggested sequence | Ser | S-DNP-Cys | Thr | Thr | Asn |
|---|---|---|---|---|---|
| Amino acid ratio* | 1.00 | 0.75 | | 1.90 | 1.05 |
| Method of sequence determination** | → | | ← | ← | ← |

* The ratio of amino acids in the peptide was determined by quantitative amino acid analysis (average of 3 runs). S-DNP-Cys was determined from the radioactivity of the sample applied on the analyzer.

** (——) indicates a determination by amino acid composition; (→) represents an *N*-terminal determination by the Dansyl chloride procedure and (←) a *C*-terminal determination using carboxypeptidase A.

The dissolved labeled protein (420 mg) was exhaustively digested with 42 mg of pepsin during 22 hr at 30°. The reaction mixture was freeze dried, dissolved in about 5 of 2% acetic acid, and applied on a Sephadex (G-25) column (fig. 1). The fractions marked by the shaded area (S) were pooled, freeze dried, redissolved in about 3 ml of 0.2 M pyridine acetate, pH 3.3, and applied on a Dowex 50-X8 column (fig. 2). The major radioactive peak (SD, shaded area) was pooled, evaporated in high vacuum, dissolved in 1.5 ml of 2% acetic acid and applied on paper for preparative chromatography (fig. 3). The radioactive peptide (total yield 30.5%) was eluted from the paper with 2% acetic acid and subjected to structural analysis (table 1).

## 4. Discussion

The results reported in this communication demonstrate that dinitrophenylation of protein sulfhydryls is not merely a mild reversible method for labeling cysteines [2] but has also the advantage that the label is bound strongly enough to allow the exact identifi-

cation of the labeled locus in the polypeptide chain. It is hoped that this combination of properties will make dinitrophenylation useful for differential labeling of proteins i.e. for the masking "super reactive" groups while labeling other, less reactive but functionally important groups in the protein.

This paper also provides direct evidence supporting our suggestion [2] that the stoichiometric inactivation of apo-GAPD with FDNB (pH 8.0 and 22°) is due to the labeling of a unique cysteine in the peptide chain. This cysteine residue was found to be located in the sequence Ser–Cys–Thr–Thr–Asn which corresponds to cysteine number 149 in GAPD from pig skeletal muscle [11]. The same residue was preferentially labeled previously with iodoacetic acid [12] and with *N*-(dimethylamino-3,5-dinitrophenyl)-maleimide [13]. However, neither one of these labeling procedures is reversible. S-DNP-apo-GAPD appears to be therefore a more appropriate derivative of GAPD for studies attempting to elucidate the contribution of the above mentioned cysteine residue to the structure of the enzyme and its functions (binding of $NAD^+$ or substrates, subunit interactions etc.). Structural and functional changes observed on such a derivative can be attributed to the modification itself, not to other irreversible side reactions triggered by the chemical modification. It should be emphasized however that such a method cannot exclude the possibility that the changes observed are due to reversible alterations in the conformation, resulting from the introduction of the label.

The exact location of the cysteine residue labeled by FDNB is also of importance for the identification of intramolecular crosslinks introduced into GAPD by 1,5-difluoro-2,4-dinitrobenzene [14] a reagent which resembles FDNB in its size, shape and reactivity [15].

**Acknowledgement**

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